Thioglycerol Inhibition of Growth and Aflatoxin Production in *Aspergillus parasiticus*

The effects of thioglycerol on growth and aflatoxin production by *Aspergillus parasiticus* were studied using conidia-initiated cultures and pregrown mycelia. Thioglycerol inhibited both growth and aflatoxin synthesis, with toxin formation being affected to a greater extent. This inhibitory activity could not be overcome by addition of methionine or zinc sulphate. Accompanying respirometric studies suggested that the primary mode of action of thioglycerol involves an inhibition of oxygen utilization.

INTRODUCTION

Various investigators (Mateles & Adye, 1965; Davis & Diener, 1968; Buchanan & Ayres, 1977; Abdollahi & Buchanan, 1981; Buchanan & Stahl, 1984) have demonstrated that glycerol strongly supports aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*, and that it is one of the simplest compounds that can induce toxin synthesis by these species. However, relatively little research pertaining to aflatoxin formation has used glycerol as a carbon source, even though potentially it could simplify studies into catabolism-associated controls regulating aflatoxin synthesis (Abdollahi & Buchanan, 1981). To investigate how glycerol acts as an effective carbon source for aflatoxin production, studies were undertaken to evaluate thioglycerol (3-mercapto-1,2-propanediol) as a potential glycerol analogue. Thioglycerol has been reported to inhibit the growth of Gram-positive and Gram-negative bacteria (Jensen & Javor, 1981); however, there appear to be no reports concerning its activity in fungi. The objective of this investigation was to characterize the effects of thioglycerol on growth and aflatoxin production by *A. parasiticus*.

METHODS

Micro-organism. Aspergillus parasiticus NRRL 2999 was maintained on potato dextrose agar (Difco) slants stored at 4 °C, and transferred monthly. Spore suspensions were prepared as described previously (Tice & Buchanan, 1982).

Media. YES medium (Davis *et al.*, 1966) consisted of 2% (w/v) yeast extract + 6% (w/v) sucrose. Glucose–mineral salts (GMS), peptone–mineral salts (PMS) and glycerol–mineral salts (GyMS) media were prepared by combining 60 g of the appropriate carbon source with 10 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 2 g $MgSO_4$, 1 ml metal mix (Buchanan *et al.*, 1983) and 1000 ml H_2O. The media were adjusted to pH 4·5 or 7·0 using 10 M-HCl or NaOH.

Culture techniques. The effects of thioglycerol on growth and aflatoxin production by conidia-initiated cultures of *A. parasiticus* at two initial pH values were studied using YES, GMS, PMS and GyMS. The media were prepared at 1·25 times required strength, adjusted to the appropriate pH, transferred in 20 ml portions to 125 ml Erlenmeyer flasks, and sterilized by autoclaving. Appropriate amounts of filter-sterilized thioglycerol stock solution (100 mg ml^{-1}) and sterile water were then added to achieve the desired thioglycerol concentration and a

total culture volume of 25 ml. Each flask was inoculated with 0·5 ml of spore suspension to achieve an inoculum of $2{\cdot}0 \times 10^4$ conidia ml^{-1}. All flasks were incubated without agitation for 7 d at 28 °C; pH, aflatoxin production and mycelium dry weight were then determined.

The effect of thioglycerol on aflatoxin production by pregrown mycelia was studied using the sequential replacement culture technique of Buchanan & Lewis (1984*b*). The mould was initially cultured in YES; the mycelial pellets were then homogenized in a blender and the mycelia recultured in PMS. Next, the mycelia were transferred in 2·0 g portions (wet weight) to 50 ml flasks containing 10 ml GMS with 0, 20 and 60 mg glucose ml^{-1} and 0, 10, 20, 30 and 40 mg thioglycerol ml^{-1}. After gentle mixing, all flasks were incubated without agitation for 48 h at 28 °C; pH, aflatoxin production and mycelium dry weight were then determined.

Respirometry. The effect of thioglycerol on oxygen uptake and carbon dioxide production by pregrown mycelia resuspended in GMS or GyMS (pH 4·5) was determined as described by Buchanan *et al.* (1985*a*).

Analyses. After determining pH, each culture was extracted three times with 20 ml portions of chloroform. The three extracts were then pooled and concentrated with a rotary evaporator. Aflatoxins (B_1, B_2, G_1 and G_2) were separated by thin-layer chromatography (Buchanan *et al.*, 1985*b*) on silica gel plates eluted with chloroform/acetone/water (93 : 7 : 1, by vol.) and quantified using a fluorodensitometer (model CS-930, Shimadzu). When necessary, interfering fluorescent pyrazines (Buchanan & Houston, 1982) were eliminated by re-eluting the plates with anhydrous diethyl ether. The lower limit of detection was approximately 10 ng aflatoxin per culture. After extraction, mycelium dry weights were determined gravimetrically by collecting the mycelia on preweighed filter papers and drying them for 24 h at 85 °C.

RESULTS AND DISCUSSION

Conidia-initiated cultures

The effects of thioglycerol on growth and aflatoxin production (B_1, B_2, G_1 and G_2) by *A. parasiticus* were initially evaluated using conidia-initiated cultures in YES, GMS, GyMS and PMS (Table 1). An overall pattern of inhibition was observed, with quantitative differences noted. The growth of the mould was less sensitive to thioglycerol when grown in media containing a sugar as the primary or sole carbon source (YES and GMS). In GMS and YES, thioglycerol was more inhibitory at an initial pH of 4·5, and aflatoxin production was more strongly affected than growth. Thioglycerol at 5 mg ml^{-1} completely inhibited growth in GyMS and PMS, at either initial pH. Aflatoxin production was not observed in PMS, which is in agreement with previous studies (Buchanan *et al.*, 1985*a*). In GyMS at an initial pH of 7·0, aflatoxin production was partially inhibited by 1 mg thioglycerol ml^{-1} without any corresponding inhibition of growth. In contrast, this concentration of thioglycerol strongly stimulated aflatoxin production in GyMS at an initial pH of 4·5.

Replacement cultures

The sequential culturing of *A. parasiticus* in YES and PMS, followed by the transfer of the disrupted mycelia to suitable replacement media, has been successfully used in our laboratory to evaluate factors influencing aflatoxin synthesis while minimizing effects due to growth differentials (Buchanan & Lewis, 1984*b*; Buchanan *et al.*, 1985*a*, *b*). This technique was used to evaluate the effect of thioglycerol on aflatoxin production. Replacement cultures containing various combinations of glucose and thioglycerol at two pH values were used.

In all cases, increasing concentrations of thioglycerol produced a dose-related decrease in aflatoxin production, mycelial mass and final pH (Table 2). The low aflatoxin production consistently observed in the cultures without glucose has been attributed largely to carry-over of toxin formed during the initial growth of the mould in YES (Buchanan *et al.*, 1985*a*, *b*). In the current study thioglycerol decreased aflatoxin production in the replacement cultures without glucose, suggesting that thioglycerol either inhibited a small amount of *de novo* toxin formation or stimulated the degradation of carried-over preformed toxin. In the replacement cultures containing 20 and 60 mg glucose ml^{-1}, thioglycerol strongly inhibited toxin production: 10 mg thioglycerol ml^{-1} reduced aflatoxin production to that observed with the controls lacking glucose (i.e. little or no *de novo* synthesis). The effect was approximately the same at both initial pH values.

Table 1. *Effect of thioglycerol in various media on growth and aflatoxin production by A. parasiticus cultures initiated from conidia*

Medium	Initial pH	Thioglycerol concn (mg ml^{-1})	Mycelium dry wt (mg)	Final pH	Aflatoxin content: Per culture (μg)	Aflatoxin content: Mycelium (ng mg^{-1})
YES*	4·5	0	369 (9)	7·0	1492·4 (43·1)	4044
		1	411 (13)	3·6	1110·8 (74·0)	2703
		5	89 (10)	4·2	<0·1	<1
		10	NG	4·3	0·0	–
		20	NG	4·4	0·0	–
	7·0	0	251 (2)	7·0	234·7 (20·9)	935
		1	237 (17)	7·3	234·3 (6·4)	989
		5	279 (4)	5·0	259·4 (11·9)	930
		10	40 (11)	6·4	0·0	0
		20	NG	6·5	0·0	–
GMS*	4·5	0	420 (18)	2·5	50·5 (6·4)	120
		1	358 (29)	2·8	68·9 (10·6)	192
		5	127 (17)	3·9	0·0	0
		10	NG	4·3	0·0	–
		20	NG	4·4	0·0	–
	7·0	0	442 (43)	4·0	160·1 (28·6)	362
		1	425 (45)	3·8	165·8 (52·2)	390
		5	210 (37)	4·9	37·6 (26·3)	179
		10	NG	5·8	0·0	–
		20	NG	6·1	0·0	–
GyMS†	4·5	0	187 (16)	2·5	28·7 (3·2)	161
		1	195 (18)	2·6	220·3 (27·7)	1190
		5	NG	4·5	0·0	–
		10	NG	4·5	0·0	–
		20	NG	4·5	0·0	–
	7·0	0	141 (25)	4·1	294·3 (57·9)	2029
		1	137 (21)	3·3	41·0 (3·6)	397
		5	NG	6·9	0·0	–
		10	NG	6·9	0·0	–
		20	NG	6·9	0·0	–
PMS*	4·5	0	253 (16)	7·7	0·0	0
		1	219 (11)	7·6	0·0	0
		5	NG	4·5	0·0	–
		10	NG	4·6	0·0	–
		20	NG	4·6	0·0	–
	7·0	0	110 (6)	8·0	0·0	0
		1	115 (16)	7·9	0·0	0
		5	NG	7·0	0·0	–
		10	NG	6·9	0·0	–
		20	NG	6·9	0·0	–

NG, No growth.

* Values for mycelium dry wt, final pH and aflatoxin content are means (± SEM) of results from four replicate cultures.

† Values for mycelium dry wt, final pH and aflatoxin content are means (± SEM) of results from eight replicate cultures.

When glycerol was used as carbon source in the replacement culture medium (Table 3), the inhibitory activity of thioglycerol was similar to that noted with the GMS replacement cultures (Table 2). The equivalent effects in GMS and GyMS indicate that thioglycerol is not competitively inhibiting the utilization of glycerol, and it is unlikely that thioglycerol is acting as a glycerol analogue. The stimulation of aflatoxin production observed in the conidia-initiated GyMS cultures containing 1 mg thioglycerol ml^{-1} at an initial pH of 4·5 (Table 1) was not

Table 2. *Aflatoxin production by replacement cultures of A. parasiticus transferred from PMS to GMS containing various combinations of glucose and thioglycerol*

Initial pH	Glucose concn (mg ml^{-1})	Thioglycerol concn (mg ml^{-1})	Mycelium dry wt* (mg)	Final pH*	Aflatoxin content (per culture)* (μg)
4·5	0	0	290	7·7	4·5 (0·3)
		10	240	6·1	1·9 (0·6)
		20	230	5·0	1·2 (0·6)
		30	220	4·9	0·7 (0·1)
		40	240	5·2	0·4 (0·1)
	20	0	360	6·8	65·6 (6·2)
		10	270	4·5	3·9 (1·2)
		20	260	4·5	1·1 (0·2)
		30	250	4·6	0·6 (0·1)
		40	190	5·0	0·1 (0·1)
	60	0	490	3·0	145·3 (3·7)
		10	190	4·8	4·1 (0·2)
		20	180	4·9	2·7 (0·8)
		30	190	5·0	0·8 (0·2)
		40	190	4·9	0·5 (0·1)
7·0	0	0	220	7·4	5·6 (0·8)
		5	190	6·7	5·0 (1·3)
		10	180	6·5	4·3 (1·0)
		20	180	6·4	2·2 (0·4)
		40	150	6·7	0·9 (0·1)
	20	0	240	6·8	48·3 (5·6)
		5	250	5·8	7·0 (0·3)
		10	190	5·7	4·0 (0·9)
		20	180	5·9	1·9 (0·1)
		40	190	6·6	1·3 (0·3)
	60	0	320	4·5	283·1 (32·8)
		5	280	4·9	104·9 (15·4)
		10	210	5·8	2·6 (1·0)
		20	200	6·4	1·8 (0·2)
		40	220	6·4	0·8 (0·2)

* Values are means (± SEM) of results from three replicate cultures.

detected in the GyMS replacement cultures. The reason for this difference is unknown. Overall, the results of the replacement culture studies indicate that thioglycerol directly inhibits aflatoxin synthesis, and that this effect can be separated from its effects on growth.

Respirometry

Javor (1983*a*, *b*) reported that the inhibition of *Escherichia coli* by thioglycerol appeared to involve two sites of action – inhibition of respiration and depression of intracellular *S*-adenosylmethionine (SAM) levels – and could be largely overcome by anaerobic incubation. In order to evaluate any role of altered respiratory activity in the inhibition of aflatoxin production by thioglycerol, its effects on oxygen uptake and carbon dioxide production by *A. parasiticus* were determined using GMS and GyMS as the replacement culture media.

Thioglycerol inhibited both oxygen uptake and carbon dioxide production in GMS replacement cultures in a dose-dependent manner (Fig. 1*a*). At the lower thioglycerol concentration, oxygen uptake was inhibited more than carbon dioxide production. This suggests that thioglycerol affected the oxidative utilization of glucose more than the fermentative catabolism of the sugar. This would be consistent with thioglycerol acting at least in part at a mitochondrial locus.

Table 3. *Aflatoxin production by replacement cultures containing various combinations of thioglycerol and glycerol*

Initial pH	Glycerol concn (mg ml^{-1})	Thioglycerol concn (mg ml^{-1})	Mycelium dry wt* (mg)	Final pH*	Aflatoxin content (per culture)* (μg)
4·5	0	0	210 (14)	6·5	5·7 (0·1)
		1	212 (8)	6·2	5·1 (0·2)
		5	196 (3)	5·7	3·5 (1·4)
		10	157 (7)	5·3	0·5 (0·1)
		20	155 (8)	5·4	1·8 (0·3)
	20	0	317 (15)	2·9	63·3 (17·5)
		1	279 (15)	2·9	80·1 (11·0)
		5	216 (9)	3·9	2·0 (0·5)
		10	174 (4)	4·8	1·8 (0·6)
		20	149 (2)	4·9	0·8 (0·2)
	60	0	427 (29)	2·5	185·1 (47·1)
		1	362 (17)	2·5	157·9 (27·5)
		5	215 (7)	3·5	4·1 (0·3)
		10	177 (3)	4·7	4·8 (0·1)
		20	164 (8)	4·9	1·0 (0·3)
7·0	0	0	197 (13)	7·5	3·9 (0·1)
		5	188 (8)	6·8	5·0 (0·3)
		10	196 (5)	6·7	4·2 (0·6)
		20	180 (6)	6·7	2·9 (0·4)
		40	176 (7)	6·7	1·5 (0·2)
	20	0	267 (8)	6·9	27·0 (3·6)
		5	204 (14)	5·9	5·1 (0·4)
		10	192 (5)	6·4	3·5 (0·1)
		20	185 (7)	6·5	2·4 (1·2)
		40	177 (3)	6·5	0·6 (0·1)
	60	0	322 (8)	5·5	136·4 (42·6)
		5	226 (17)	6·5	5·9 (1·7)
		10	202 (5)	6·4	3·7 (0·6)
		20	229 (31)	6·4	2·0 (0·9)
		40	220 (17)	6·4	0·7 (0·1)

* Values are means (± SEM) of results from three replicate cultures.

Thioglycerol inhibited respiration in the GyMS cultures in a manner similar to that noted with the GMS cultures, except that the amount of carbon dioxide evolved per unit of oxygen consumed was significantly less in both the control and 5 mg thioglycerol ml^{-1} cultures (Fig. 1*b*). Further, both oxygen and carbon dioxide utilization declined at a substantially slower rate in the GyMS cultures during the latter stages of the post-transfer incubation. These differences probably reflect differences in the catabolism of the carbon sources; it would appear that the mould utilizes glycerol predominately in an oxidative manner.

Previous investigations (Buchanan & Lewis, 1984*a*; Buchanan *et al.*, 1985*a*) have indicated that, at least in *A. parasiticus* NRRL 2999, maximal aflatoxin synthesis correlates with a period of increased respiratory activity, and that if this is blocked aflatoxin accumulation is depressed. This suggests that a potential means of decreasing aflatoxin production in stored agricultural products is to identify appropriate environmental or chemical means for depressing respiration in the mould. Aflatoxin production is greatly reduced in materials stored under partial anaerobiosis (Wilson *et al.*, 1975; Clevstrom *et al.*, 1983).

Supplementation with methionine and zinc

The depression of SAM levels in *E. coli* can be at least partially reversed by methionine supplementation (Javor, 1983*a*). However, methionine supplementation (1, 5 and 10 mg ml^{-1}) of conidia-initiated and replacement cultures did not affect inhibition of aflatoxin production by

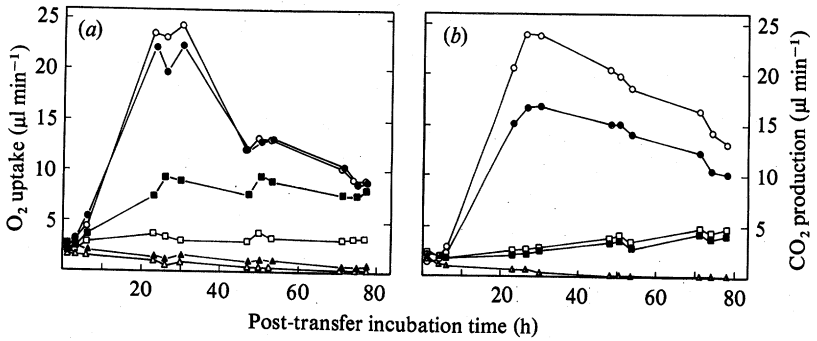

Fig. 1. Oxygen uptake and carbon dioxide production by replacement cultures of *A. parasiticus* after transfer from PMS to GMS (*a*) or GyMS (*b*) containing 0 (○, ●), 5 (□, ■) or 20 (△, ▲) mg thioglycerol ml^{-1}. ○, □, △, Oxygen uptake; ●, ■, ▲, carbon dioxide production. Values are means of results from three replicate cultures.

Table 4. *Effect of zinc supplementation on the inhibition of A. parasiticus by thioglycerol*

Thioglycerol concn (mg ml^{-1})	Additional zinc sulphate concn* (mg ml^{-1})	Mycelium dry wt† (mg)	Aflatoxin content†	
			Per culture (μg)	Mycelium (ng mg^{-1})
0	0·0	311 (20)	43·4 (4·4)	140
	0·1	283 (9)	52·4 (17·4)	185
	1·0	229 (10)	105·5 (19·1)	460
1	0·0	230 (4)	95·1 (30·4)	413
	0·1	254 (24)	54·0 (12·4)	213
	1·0	139 (16)	72·6 (17·4)	522
2	0·0	203 (11)	138·1 (8·9)	680
	0·1	184 (7)	139·5 (15·1)	758
	1·0	29 (2)	4·3 (1·9)	148
5	0·0	16 (3)	0·4 (0·3)	25
	0·1	19 (5)	0·0	0
	1·0	16 (1)	0·0	0

* The basal concentration of zinc sulphate was 1·8 μg ml^{-1}; the initial pH was 4·5.
† Values are means (± SEM) of results from three replicate cultures.

thioglycerol (data not shown). This suggests that depression of SAM levels is not a major factor associated with the activity of thioglycerol against *A. parasiticus*.

2-Mercaptoethanol, a compound which can be considered a structural analogue of thioglycerol, inhibited growth and aflatoxin production by *A. parasiticus*. (Gupta *et al.*, 1976). It was suggested that this was due to the chelation of zinc, a trace mineral essential for aflatoxin synthesis. To ascertain if thioglycerol could be acting in such a manner, conidia-initiated GMS cultures were supplemented with various combinations of thioglycerol and zinc sulphate. Increasing the zinc content of the medium did not overcome the inhibitory effects of thioglycerol (Table 4) indicating that thioglycerol does not act via zinc chelation.

In summary, thioglycerol inhibits both growth and aflatoxin production by *A. parasiticus*, with toxin production being affected more strongly. This does not appear to be due to thioglycerol acting as a glycerol analogue, nor does it involve alterations in intracellular SAM concentrations or zinc chelation. Instead, thioglycerol appears to be affecting *A. parasiticus* by inhibiting mitochondrial respiratory activity. This is similar to the findings of Javor (1983*b*) who hypothesized that in *E. coli* the primary mode of action of thioglycerol involves an inhibition of cytochrome oxidase.

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